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13. ABSTRACT *(Maximum 200 Words)*

Award DAMD17-99-1-9367 seeks to understand the role of membrane trafficking in Epidermal Growth Factor Receptor (EGFR) signal transduction. We have been using a tissue culture model system (HeLa cells) to isolate the activated EGFR at distinct stages in the endocytic pathway. Our research has focused on rab5, a small molecular weight GTPase, implicated in the biogenesis of the early endosome. Mutations to modulate the guanine nucleotide binding properties of this protein have been reported for constituitvely internalized receptors, but little is known about its role in EGFR endocytic trafficking. In the past year, we have published our finding that constituitively active rab5 (rab5(Q79L)) induced enlarged early endosomes without any consequence on receptor or membrane trafficking (Ceresa, et. al, JBC 276 p. 9649). Our continued pursuit on this avenue of research has shown that the dominant negative form of rab5 (rab5(S34N)) does not block EGFR endocytosis, but may block entry on the EGFR into the early endosome. We are in the process of developing a biochemical assay to confirm our immunofluorescence observations. Additionally, we are in the process of developing a model system to examine the role of endocytic trafficking on signaling by other members of the ErbB family, namely ErbB2.

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Introduction:

The signal transduction specificity that underlies Epidermal Growth Factor (EGF) Receptor (EGFR) physiology is an important component of tissue growth and development. Improper regulation of signal transduction by the EGFR and related family members is characteristic of many mammary carcinomas. Identifying the basic molecular mechanisms that regulate this signaling network is an important step in distinguishing the difference between positive (normal growth) and negative (uncontrolled cell proliferation) EGFR-mediated cell biology. A well described phenomenon that accompanies EGFR signaling is the entry of the activated EGFR into the cell via clathrin coated vesicles (1). Inhibition of EGFR internalization results in the selective inhibition of some, but not all, signaling pathways (2-5). The purpose of this research is to further explore the spatial and temporal components of EGFR signal transduction by examining the signaling properties of the activated EGFR at various points in the endocytic pathway. The scope of this research is to identify membrane trafficking proteins involved in EGFR endocytosis and to use mutant forms of these membrane trafficking proteins to develop a model system that permits isolation of the EGFR at discrete endocytic stages. Our long-term goal is to determine the biochemical characteristics and the signaling properties of the EGFR, and other members of the EGFR family, namely ErbB2, at these discrete endosomal stages.

Body:

Growth Factors and their corresponding cell surface receptors are important components in the maintenance and growth of normal epithelial cells. Overexpression and unregulated signaling of members of the ErbB growth factor receptor family is associated with many mammary carcinomas and a poor prognosis for recovery (6,7). This proposed research seeks to better understand the coordinated regulation of signaling by the ErbB family of cell surface receptors, and more specifically, ErbB1 or the Epidermal Growth Factor (EGF) Receptor (EGFR). The EGFR, like many cell surface receptors, undergoes ligand-mediated endocytosis. This internalization process has historically been thought to be merely a mechanism for attenuating receptor signaling by removing the activated receptor and ligand from the cell surface and dissociating the ligand/receptor complex. It is now appreciated that the role of the endocytic pathways plays a complex regulatory role in EGFR signaling (2-5, 11). Our hypothesis is that each stage in the endocytic pathway uniquely regulates EGFR signaling. The goal of our research is to overexpress proteins involved in endocytic trafficking to disrupt normal endocytic trafficking of the EGFR allowing for analysis of EGFR signaling at distinct cellular locations.

Specific Aim I

Rab5 is a small molecular weight GTP-binding protein that has been well described as an important regulator in the formation of the early endosome (8,9). A point mutation of rab5 (glutamine to lysine mutation at residue 79 – denoted Q79L) reduces the ability of rab5 to hydrolyze GTP thus, leaving the protein in the constituitively active state. The consequence of this mutation is an enlarged early endosome enriched in transferrin receptors (9,10). From our initial experiments, we concluded that while rab5(Q79L) can induce the formation of an enlarged

early endosome, the formation of this enlarged endosome does not accurately predict of changes in the kinetics of transferrin receptor uptake and/or recycling (12). An examination of the trafficking of the EGFR revealed that expression of rab5(Q79L) did not alter its trafficking kinetics either. We have also examined rab5(Q79L)'s effects of EGFR trafficking in HepG2 cells, and found trafficking is not altered in other cellular systems (13). Taking these studies as a whole, we concluded that rab5 is not the rate limiting step in regulation of EGFR trafficking to the early endosome.

Surprisingly, it was reported in the literature that overexpression of dominant negative rab5 (rab5(S34N)) inhibits the rate of EGFR endocytosis (14). This lead us to re-examine our first specific aim, using another angle to examine the role of rab5 in EGFR endocytosis. Toward this end, we generated a tetracycline-regulatable adenovirus that encodes for HA-rab5(S34N). When rab5(S34N) adenovirus is expressed in tTA-HeLa cells, there is no change in the rate of EGFR endocytosis (as measured by ^{125}I-EGF uptake) (Figure 1) or fluid phase uptake (Figure 2). Confocal microscopy confirmed that cells expressing HA-rab5(S34N) internalize fluorescently-labeled EGF (Figure 1, Appendix). A more detailed examination of these images, revealed that unlike rab5(WT) and rab5(Q79L) expressing cells, the internalized, fluorescently-labeled EGF in rab5(S34N) expressing cells did not co-localize with the exogenous rab5. These data suggest to us that expression of dominant negative rab5 allows the internalization of EGF, but does not permit its delivery to the early endosome.

Currently, we are in the process of developing a biochemical assay that will accurately determine if the expression of HA-rab5(S34N) inhibits the entry of endocytic cargo into the early endosome. Our hypothesis is that import of cargo into the early endosome can be blocked by expression of the dominant negative HA-rab5(S34N). Currently, we are in the process of

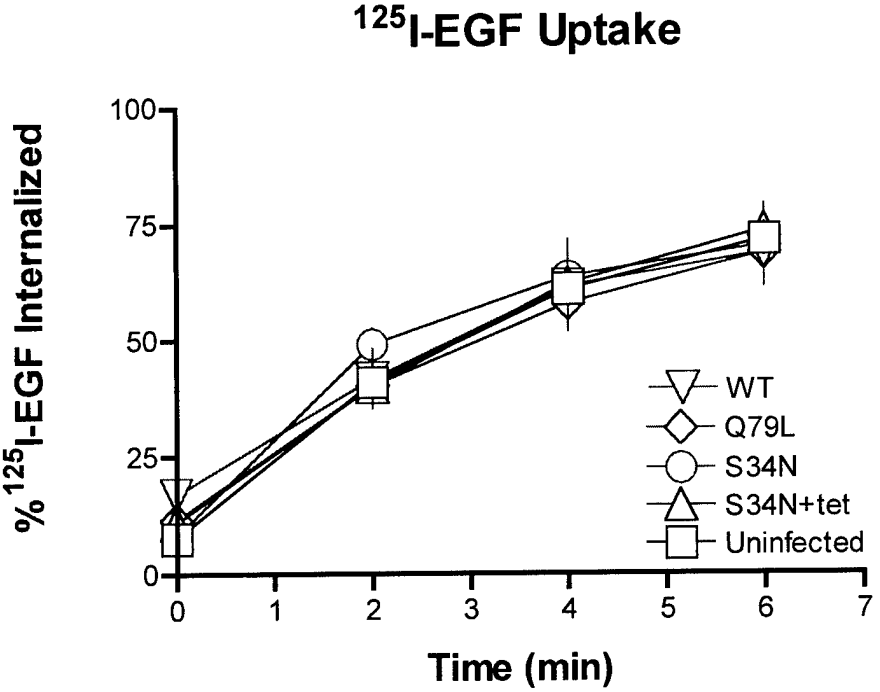

Figure 1. Overexpression of rab5(WT), rab5(Q79L), or rab5(S34N) has no effect on the rate of ^{125}I-EGF internalization. Subconfluent 35 mm dishes of tTA-HeLa cells were infected for 2 hours with adenoviruses encoding for the indicated rab5 mutants. Cells were returned to growth media for 18 hours and then assayed for ^{125}I -EGF internalization. Briefly, the infected cells were incubated on ice for 2 hours with XX nM ^{125}I -EGF (ci/mol) to achieve steady-state binding of cell surface EGFR. Free radioligand was removed with a series of washes using ice cold PBS. The cells were incubated with pre-warmed 37°C media for the indicated amounts of time, allowing for internalization of the radioligand/receptor complex. Dissociated ligand was removed with a series of ice cold PBS washes. Cell surface ^{125}I -EGF was measured as the amount of acid dissociatable radioligand (determined as the amount of radioactivity disociated in two 8 min washes in 0.2M NaCl/0.2 M acetic acid) and internalized ^{125}I –EGF was assessed from the remaining cell associated radioacitivty (assayed by solubilizing the remain cells in 1.0 M NaOH). Data are plotted as the percent internalized (cpm internalized/(cpm cell surface + cpm internalized)) at each time point. Data are plotted as the average ± S.E.M. from 3-4 experiments from each condition.

developing an assay that will permit separation of the early endosome from intermediate endosomes using centrifugation over sucrose gradients. Briefly, horseradish peroxidase (HRP) will be internalized into cells via fluid phase endocytosis. Post-nuclear supernatants from broken cell preparations will be layered over sucrose gradients and centrifuged for 15 hours. Sucrose gradients will be fractionated into 0.5 ml fractions. Individual fractions will be assayed for HRP enzymatic activity using a colorimetric assay and the presents of rab5 will be determined by western blot. The co-migration of rab5 and HRP will be determined in cells expressing endogenous rab5 as well as those expressing exogenous rab5(WT), rab5(Q79L), or rab5(S34N).

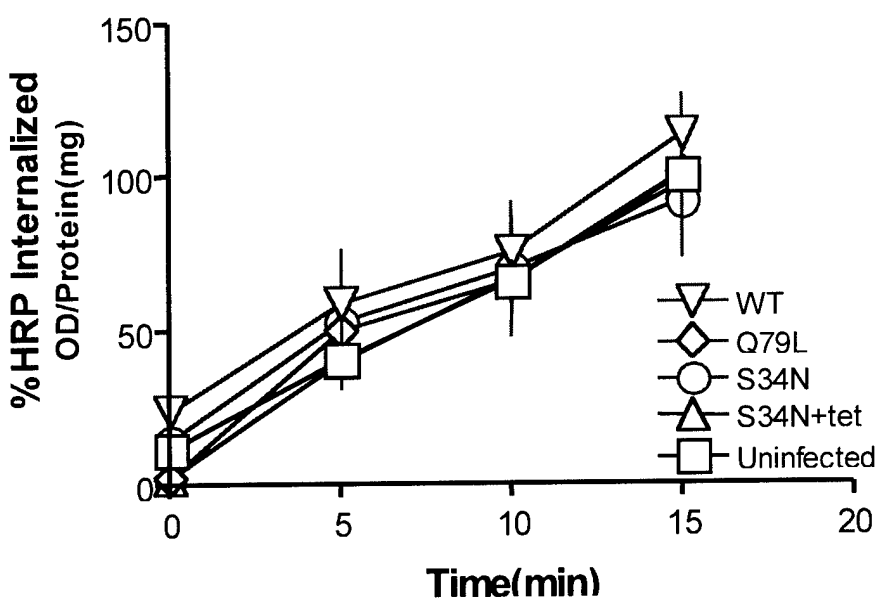

Figure 2. **Overexpression of rab5(WT), rab5(Q79L), or rab5(S34N) has no effect on the rate of fluid phase endocytosis.** 35 mm dishes of cells were infected as described in Figure 1. Fluid phase endocytosis was measured as a function of HRP uptake. Cells were incubated with 4 mg/ml HRP at 37°C for the indicated times. Uptake was terminated by six 5 minute washes in PBS/1 mM $MgCl_2$/1 mM $CaCl_2$/0.2% BSA, treatment with 0.1% pronase to remove extracellular HRP and centrifuged through a 0.5M sucrose cushion. Cells were solublized in a 0.5 % triton solution and assayed for HRP using O-phenylenediamine as a substrate, and quantified by measuring the O.D. at 490 nm. A fraction of the sample was assayed for protein concentration. Data have been normalized to uninfected cells. The ratio of O.D. to protein have been normalized to the 10 minute time point in unifected cells and plotted as a function of time. Shown are the average ± S.E.M. from 2-4 experiments for each condition.

In summary, our pursuit of understanding the endocytic trafficking of the EGFR has allowed us the unexpected discovery that expression of rab5(S34N) can disrupt ligand-mediated EGFR entry into the early endosome without perturbing EGFR endocytosis. While these results are discrepant to other published results, they are consistent with in vivo models and our prior findings. We are in the process of completing these experiments and anticipate submitting our findings for publication to either the Journal of Cell Biology or the Journal of Biological Chemistry this fall.

These data were obtained by deviating slightly from our initial proposed statement of work. However, conceptually the long-term goals of the project have not changed. We remain committed to developing a model system for the study of EGFR-mediated signaling mechanism

during the course of endocytosis. If our data with the HA-rab5(S34N) stand up under further examination, we will have developed a model system that disrupts EGFR trafficking a discrete cellular location.

Specific Aim II

Specific Aim II tests the hypothesis that EGFR signaling is regulated by the cellular location of the EGFR. The strategy we will use for these studies will be detailed dose response and time course studies comparing the EGFR signaling in cells with differing endocytic trafficking properties. We had planned to contrast EGFR signaling from the plasma membrane (expressing dominant negative dynamin), the early endosome (expressing constituitively active rab5), and HeLa cells (expressing no exogenous proteins). Our progress on this specific aim has been impeded by the unexpected finding that HA-rab5(Q79L) expression does not alter EGFR uptake or recycling. We feel it is prudent that we understand the role of rab5 in the endocytic pathway of the EGFR before we embark upon this specific aim. Upon completion of our current investigation of rab5(S34N), we will be well suited to begin these studies.

Specific Aim III

Specific Aim III examines the signaling properties of ErbB2, a member of the EGFR family of recpetor tyrosine kinases with no known ligand. While considerably more difficult to study than the EGFR, ErbB2 has proven to be an effective target for some chemotherapuetics. Currently, we are in the process of establishing an appropriate modeling system for the study of this receptor. We have wild type and mutant forms of dynamin and rab5 that express in tetracycline regulatable adenoviral expression vectors. This allows us the versatility to study the effects of these membrane trafficking altering proteins in a number of cell lines. We have identified a number of candidate cells lines (HeLa, MCF-7, and HepG2 cells) and are in the

process of characterizing their basal number of ErbB2 receptors as well as the contribution of the ErbB2 receptor in signaling.

- **Key Research Accomplishments**:

- Characterization of rab5(WT) and rab5(Q79L) in tTA-HeLa cells. Reported in Ceresa, B.P., et. al. *J Biol Chem* 276 (13) p 9649-9654).
- Characterization of rab5(WT) and rab5(Q79L) in tTA-HepG2 cells; the effect of rab5(Q79L) on EGFR trafficking. Reported in Ceresa, B. P. and Schmid, S.L., 2001 AACR abstract #5023.
- Generation and characterization of rab5(S34N) adenovirus in tTA-HeLa cells.
- Demonstration that expression of rab5(S34N) does not inhibit EGFR or fluid phase endocytosis, but does inhibit entry into the early endosome.
- Initiation of studies that examine EGFR-mediated signal transduction from a pre-endosomal compartment.

- **Reportable Outcomes**

Manuscripts:

- B. P. Ceresa, M. Lotscher, and S. L. Schmid, Receptor and Membrane Recycling can Occur with Unaltered Efficiency Despite Dramatic Rab5(Q79L)-induced Changes in Endosome Geometry (2001) *J Biol Chem* 276 (13) p 9649-9654.

Abstracts:

B. P. Ceresa, S. L. Schmid, Expression of rab5(Q79L)-induced Enlarged Endosomes Does Not Alter Epidermal Growth Factor Receptor Endocytic Trafficking. Abstract # 5023 at 2001 Annual Meeting of the American Association of Cancer Researchers, New Orleans, LA.

Cell lines Developed:

Human hepatoma cells (HepG2 cells) were stably transfected to express the tetracycline transactivator – tTA-HepG2. By stably expressing the tetracycline transactivator, we can express our rab5 adenoviruses that are under the control of a tetracyline inducible promoter. This new cellular environment will allow us to examine the consequence of the various rab5 mutants on the endocytic trafficking of endogenous EGFR.

Adenoviruses:

We have continued to develop a number of adenoviruses that express proteins that are involved in membrane trafficking. We have generated tetracycline-regulatable adenoviruses that express a dominant negative rab5 (HA-rab5(S34)), and wild type, dominant negative, and constituitively active rab7 (HA-rab7(WT), HA-rab7 (N125I), and HA-rab7(Q67L), respectively).

Conclusions:

In the past year we have made tremendous headway on understanding the role of rab5 in the endocytic trafficking of the EGFR. Expression of a constituitively active mutant of rab5 (rab5(Q79L)) results in the formation of an enlarged endosome but does not alter the membrane or receptor trafficking (12, 13), thus we conclude rab5 is not the rate limiting step in the endocytic trafficking of the EGFR. To more thoroughly pursue this question, we developed an adenovirus expressing dominant negative rab5 (rab5(S34N)). Our preliminary biochemical immunofluorescence data indicate that rab5(S34N) does not effect EGFR or fluid phase endocytosis. Examination of the localization of internalized EGFR reveals that expression of rab5(S34N) can block endocytic cargo into the early endosome. We are in the process of determining whether these results can be confirmed biochemically.

Taken together, these data offer new insight into the role of rab5 in EGFR endocytic trafficking. Rab5 has been well documented to play a role in the endocytosis of proteins involved in the internalization of constituitively recycled cell surface proteins (i.e. transferrin and low density lipoprotein receptors), however the role of rab5 in ligand-mediated endocytosis has not been examined. We are continuing to delinate the interplay between EGFR-mediated endocytosis and signal transduction. These studies provide important insight in to the role of rab5 in EGFR endocytosis as well as identifying a potential point of regulation for EGFR signaling. As these studies continue, we will learn if EGFR entry into the early endosome requires rab5 and if so, what the role of early endosome in EGFR-mediate physiology. This information as great impact on the identification of molecular targets for the disruption of unregulated EGFR-mediated growth characteristic of many breast cancers.

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Appendices

A. **Confocal Micrscope Image** – HA-rab5 expression and Alexa488-EGF internalization

B. **Manuscript** – Ceresa et. al. (2001), JBC 276 (13) p. 9649-9654.

C. **Abstract** – Ceresa and Schmid, (2001) AACR.

D. **List of Personnel receiving money from this grant.**

Appendix A
Figure 1

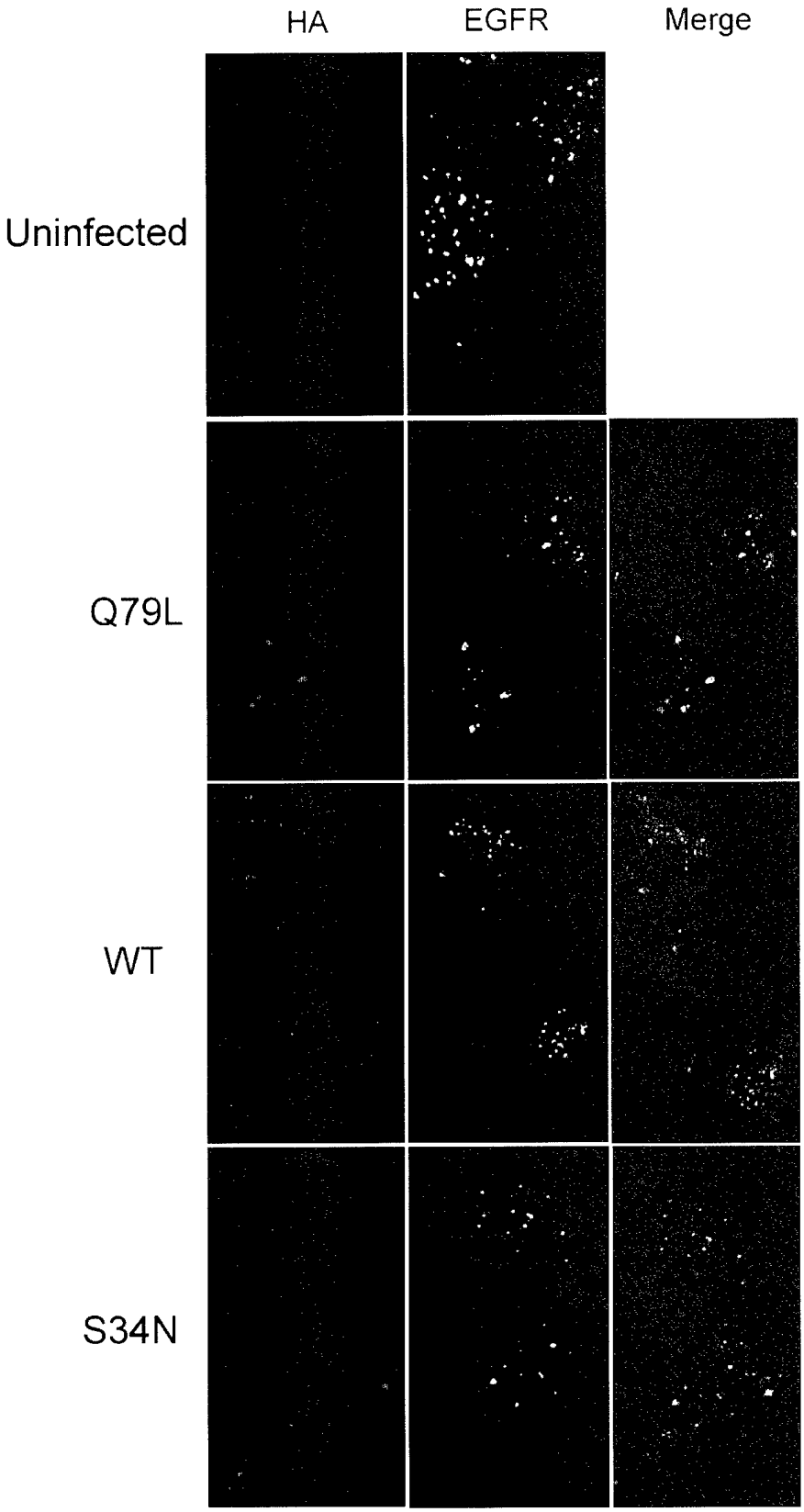

Figure 1. Fluorescently-labeled EGF can internalize in cells expressing rab5(WT), rab5(Q79L), and rab5(S34N). HeLa cells were plated on coverslips and transfected with the cDNAs encoding for the indicated exogenous HA-tagged rab5 and incubated for 10 minutes with Alexa 488-EGF in serum-free media at 37 C. Exogenous Alexa 488-EGF was removed by 2 washes in ice cold PBS supplemented with 2mM CaCl2 and 2 mM MgCl2 (PBS-+), followed by three 5 minute washes in citrate buffer (25.5 mM citric acid/24.5 mM sodium citrate/280 mM sucrose), and re-equilibrated in PBS++. The coverslips were then fixed in a 4% p-formaldehyde solution made up in PBS++, solubilized with 0.1% saponin/5% fetal bovine serum in PBS-+. Samples were subjected to indirect immunofluorescence using an anti-"HA" rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) and visualized with a goat anti-rabbit secondary conjugated to Alexa 568. Images were collected with a Leica TCS NT confocal microscope (100X objective).

Appendix B
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Receptor and Membrane Recycling Can Occur with Unaltered Efficiency Despite Dramatic Rab5(Q79L)-induced Changes in Endosome Geometry*

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Current models for sorting in the endosomal compartment suggest that endosomal geometry plays a significant role as membrane-bound proteins accumulate in tubular regions for recycling, and lumenal markers accumulate in large vacuolar portions for delivery to lysosomes. Rab5, a small molecular weight GTPase, functions in the formation and maintenance of the early/sorting endosome. Overexpression of the constitutively active form, Rab5(Q79L), leads to enhanced endosome fusion resulting in the enlargement of early endosomes. Using an adenoviral expression system to regulate the time and level of Rab5(Q79L) overexpression in HeLa cells, we find that although endosomes are dramatically enlarged, the rates of transferrin receptor-mediated endocytosis and recycling are unaffected. Moreover, despite the enlarged endosome phenotype, neither the rate of internalization of a fluid phase marker nor the rate of recycling of a bulk lipid marker were affected. These results suggest that GTP hydrolysis by Rab5 is rate-limiting for endosome fusion but not for endocytic trafficking and that early endosome geometry may be a less critical determinant of sorting efficiencies than previously thought.

Endocytic vesicles deliver their content of membrane proteins, lipids, and lumenal content to the early or sorting endosomal compartment consisting of tubular and vacuolar portions. Many receptor-ligand complexes dissociate in the mildly acidic environment of the early endosome (1). It has been proposed (1, 2) that endosomal morphology and resulting geometric considerations play a major role in controlling sorting efficiency in the early endosome. In this model, membrane proteins destined for recycling accumulate in long tubular extensions of the early endosome, which have a high surface to volume ratio. Fluid phase content including released ligands is deposited in the vacuolar portions of the early endosome, which, being spherical, approach a minimum surface to volume ratio. These vacuolar portions dissociate from tubular regions to carry their contents to late endosomes and/or lysosomes (3).

Rab5 is a small molecular weight GTPase associated with the plasma membrane and early/sorting endosomes. Rab5 controls homotypic early endosome fusion and thus functions in the formation of early endosomes (4–6). A point mutation in the GTPase domain (glutamine to leucine; denoted as Rab5(Q79L)) reduces Rab5 GTPase activity and results in a mutant Rab5 with an increased propensity to be in the active, GTP-bound state (7, 8). Expression of this constitutively active form of Rab5 enhances homotypic endosome fusion leading to the formation of enlarged early endosomes. It also has been reported that Rab5(Q79L) overexpression increases the rate of transferrin receptor uptake and decreases the rate of transferrin receptor recycling (8) although the mechanism for these effects remains obscure.

Rab5 is preferentially associated with the vacuolar portions of the early endosome (9), and Rab5(Q79L) overexpression leads to the formation of large spherical endosomes as visualized in semithick sections by electron microscopy (10). To test whether the Rab5(Q79L) effects on transferrin receptor endocytosis and recycling can be correlated with these dramatic changes in endosomal size and geometry, we utilized a tetracycline-regulatable adenoviral expression system that allows us to temporally control Rab5(Q79L) expression levels. The early endosomal compartment was dramatically enlarged in adenovirally infected HeLa cells overexpressing Rab5(Q79L). However, the presence of these enlarged endosomes did not alter the kinetics of endocytic membrane trafficking of either cell surface receptors or bulk membrane lipids. These unexpected results argue that geometric considerations may contribute to a lesser extent than previously assumed in determining the sorting and recycling efficiencies of the early endosomal compartment.

MATERIALS AND METHODS

Cell Culture—tTA-HeLa cells were cultured in DMEM[1] supplemented with 5% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin (growth medium). Wild type and mutant (canine) Rab5a constructs (a gift of M. Zerial) were tagged with the hemagglutinin epitope on the amino terminus and subcloned into pUHD expression vectors (11). HA-Rab5(WT)- and HA-Rab5(Q79L)-expressing cells were generated by cotransfecting the tTA-HeLa cells with cDNA that encodes HA-Rab5(WT) or HA-Rab5(Q79L) (10 μg) and the plasmid pBSpac (0.5 μg) by calcium phosphate transfection. Positive clones were selected by culture in 200 ng/ml puromycin, 400 μg/ml G418, and 2 μg/ml tetracycline and screened by Western blot for their abilities to express Rab5 48 h after induction by the removal of tetracycline (11).

Stably transfected cells were cultured in the presence of 1 μg/ml tetracycline. Wild type or mutant Rab5 expression was induced by

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[1] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; PBS, phosphate-buffered saline; WT, wild type; HRP, horseradish peroxidase; Tfn, transferrin; TfnR, transferrin receptor; B-XX-Tfn, biotinylated Tfn; C_6-NBD-SM, N-((6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino) hexanoyl)-sphingosyl phosphocholine.

washing out the existing tetracycline with two PBS (phosphate-buffered saline, pH 7.4) washes and incubating the cells in growth medium without tetracycline for 48 h.

Alexa-Transferrin Labeling and Immunofluorescence—Cells, grown on coverslips to ~70% confluency, were washed twice with room temperature PBS and then incubated with 50 μg/ml Alexa-transferrin (Molecular Probes) in PBS^{++} (PBS, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.2% bovine serum albumin) for the indicated times at 37 °C. Coverslips were moved to 4 °C, washed twice with ice-cold PBS^{++++} and three times with ice-cold citrate buffer, and re-equilibrated with two additional ice-cold PBS^{++++} washes (12). The coverslips were fixed in a 4% formaldehyde/PBS^{++} (PBS, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) solution at room temperature for 5 min and on ice for an additional 15 min. Excess formaldehyde was removed with 3 × 5-min washes in PBS^{++}. Cells were permeabilized in 0.1% saponin/5% goat serum/PBS^{++} for 15 min. After 3 × 5-min PBS^{++} washes, the coverslips were incubated with primary antibody for 1 h. Antibodies used (source in parentheses) were mouse monoclonal anti-HA tag 12CA5 (Ian Wilson, The Scripps Research Institute) and mouse monoclonal anti-Rab5 (Transduction Laboratories). Unbound primary antibody was removed with 3 × 5-min PBS^{++} washes, and the coverslips were incubated with the appropriate secondary antibody (noted in figure legends). Coverslips were subjected to 6 × 5-min PBS^{++} washes, rinsed in Millipore water, and mounted on a coverslip slide using Fluoromount G (EM Sciences).

Western Blot Detection of HA-Rab5—35-mm dishes of cells were washed twice in room temperature PBS and put on ice with PBS to cool to 4 °C for 5 min. Cells were then harvested in 500 μl of ice-cold solubilization buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, and 2 mM phenylmethylsulfonyl fluoride). Lysates were solubilized by gently rocking for 10 min at 4 °C and centrifuged at 14,000 rpm in an Eppendorf microcentrifuge for 10 min at 4 °C to pellet the insoluble material. Protein concentration was determined by BCA, and 100 μg of solubilized protein was run on 13% SDS-polyacrylamide gel electrophoresis mini-gel, transferred to nitrocellulose, and immunoblotted using monoclonal antibodies against Rab5 or 12CA5 as above. Proteins were detected using secondary goat anti-mouse antibodies conjugated to horseradish peroxidase and visualized using enhanced chemiluminescence.

Adenovirus Generation—HA-Rab5(WT) or HA-Rab5(Q79L) was put under the control of a tetracycline-regulatable promoter in the pAdlox vector 3′ to the Ψ5 packaging site and 5′ to the poly(A) site. Adenoviruses were generated as previously described (13). Prior to use in experiments, adenoviruses were plaque-purified to a single viral population and then amplified.

Adenoviral Infection—In experiments in which cells expressed Rab5 continuously for 18 h, tTA-HeLa cells at ~70% confluency were infected with adenovirus at an m.o.i. of 10 plaque-forming units/cell. Cells were infected with adenovirus in DMEM with or without 1 μg/ml tetracycline for 2 h at 37 °C. After infection, the viral medium was removed and replaced with growth medium with or without tetracycline.

When Rab5 expression was studied using a bolus concentration, tTA-HeLa cells at ~70% confluency were infected with adenovirus at an m.o.i. of 300 plaque-forming units/cell. Infection took place at 37 °C for 2 h in DMEM, and the adenovirus-containing medium was removed and replaced with growth medium containing 10 μg/ml cycloheximide for 3 h to prevent protein but not mRNA synthesis. Cycloheximide was washed out with three washes of DMEM, and the cells were returned to 37 °C incubation with growth medium for the indicated times, typically 1, 3, or 12 h. At all time points, the cells remained adherent to the dish without any obvious signs of toxicity or cell death.

Transferrin Uptake and Recycling—Infected cells were assayed for transferrin uptake and recycling as previously described (14) with minor modifications. To measure a single round of transferrin receptor endocytosis, harvested cells were preincubated for 1 h with 4 μg/ml B-XX-Tfn. Prior to internalization, unbound ligand was removed by three washes in ice-cold PBS^{++++} (PBS, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, and 0.2% bovine serum albumin, pH 7.4). Cells were resuspended to a concentration of 3–4 × 10^6 cells/ml and incubated at 37 °C for the indicated times. B-XX-Tfn trafficking was stopped by transferring a 50-μl aliquot of the cell suspension to an Eppendorf tube containing 750 μl of ice-cold PBS^{++++}. Internalized B-XX-Tfn was determined using an enzyme-linked immunosorbent assay-based assay as previously described (14).

Horseradish Peroxidase (HRP) Uptake—Fluid phase uptake was performed on infected 35-mm dishes of cells as previously described (15, 16).

Lipid Recycling—Lipid recycling was performed as described by Hao and Maxfield (17). Quantitation of C_6-NBD-SM in medium or in cell lysates was performed using a PerkinElmer Life Sciences fluorimeter at an excitation wavelength of 465 nm and measuring the peak height between 518 and 558 nm. Data are plotted as the C_6-NBD-SM that remains cell-associated at each time point.

RESULTS AND DISCUSSION

Expression of HA-Rab5(Q79L) Induces Enlarged Endosomes in Stably Transformed Cells—To begin to probe the mechanism of Rab5(Q79L) effects on TfnR endocytosis and recycling, we generated stably transformed cell lines expressing HA-tagged wild type Rab5 (HA-Rab5(WT)) and constitutively active HA-Rab5(Q79L) under the control of a tetracycline-responsive expression system. Using this system, stable cell lines can be generated while avoiding any deleterious effects that may result from continuously altering cellular membrane trafficking (11). Stable cell lines generated in this manner express either HA-Rab5(WT) or HA-Rab5(Q79L) in a tetracycline-regulated manner as determined by Western blotting using either the 12CA5 anti-HA antibody (not shown) or antibodies against Rab5, which reveal both the more slowly migrating recombinant HA-tagged protein and the endogenous Rab5 (Fig. 1*A*). In these stably transformed cells, recombinant HA-Rab5 is expressed at roughly equimolar levels compared with endogenous protein. Expression of Rab5(WT) at these levels did not affect endosome morphology (Fig. 1*B*, *upper left*), whereas expression of Rab5(Q79L) at these levels was sufficient to cause the expected morphological phenotype—enlarged endosomes (*upper right*). Expression of HA-Rab5(Q79L) was suppressed when cells were cultured in the presence of tetracycline (*upper middle panel*). In all cases, the endosomes were functional in that fluorescently labeled Tfn was internalized and delivered to them (Fig. 1*B*, *lower panels*). Unexpectedly, examination of the single-round uptake and recycling kinetics of Tfn revealed that despite formation of enlarged endosomes there was no perturbation of Tfn uptake in cells induced to express either WT or mutant Rab5 compared with uninduced control cells (Fig. 1*C*). Similarly, there was no change in the steady state accumulation of Tfn receptors in this endosomal compartment. These data suggested that the enlarged endosome phenotype is not predictive of defects in endocytic membrane trafficking.

Adenovirus-mediated, Tetracycline-regulated Expression of Rab5(Q79L)—Given that a threshold level of Rab5(Q79L) expression may be required to cause changes in membrane trafficking (8), it remained possible that the lack of changes in TfnR trafficking was due to low levels of exogenous protein expression. Consequently, we elected to employ an adenoviral expression system to obtain reproducibly and uniformly higher levels of overexpression. In addition, adenovirus allows for the rapid induction of high levels of protein, thus circumventing problems that may occur as a result of chronic exposure of a foreign protein to the cell. The adenoviral expression system was designed to retain the tetracycline regulation so that any potential adenovirus effects could be controlled by infection of cells in the presence of tetracycline.

When cultured under inducing conditions for 18 h in the absence of tetracycline, tTA-HeLa cells infected with recombinant adenoviruses encoding either WT or mutant Rab5 expressed 50–100-fold higher levels of the desired protein compared with endogenous Rab5 (Fig. 2*A*). Importantly, WT and mutant Rab5 expression was not detectable when infected cells were cultured in the presence of tetracycline. As expected at these high levels of overexpression, the characteristically enlarged endosomal morphology was readily apparent in cells expressing HA-Rab5(Q79L) (Fig. 2*B*). These enlarged endosomes remained accessible to internalized Tfn as indicated by the colocalization of fluorescently labeled transferrin (Alexa-Tfn) and Rab5 containing vesicles stained with an antibody

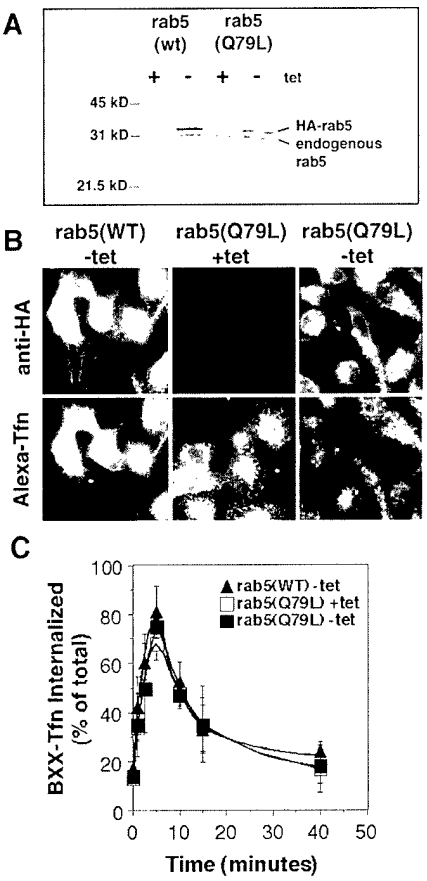

FIG. 1. **HA-Rab5(Q79L) expression in stably transformed tTA-HeLa cells causes enlarged endosomes but does not effect Tfn endocytosis or recycling.** tTA-HeLa cells stably expressing either HA-Rab5(WT) or HA-Rab5(Q79L) under control of a tetracycline (*tet*)-regulatable promoter were clonally selected as described under "Materials and Methods." Cells were incubated in the absence (induced) or presence (uninduced) of 1 μg/ml tetracycline for 48 h. *A*, immunoblots of cell lysates probed with antibodies against Rab5 showing expression of endogenous and HA-tagged Rab5. *B*, transformed tTA-HeLa cells uninduced or induced to express either HA-Rab5(WT) or HA-Rab5(Q79L) as indicated were incubated with Alexa-Tfn (*lower panels*) for 20 min at 37 °C before fixation, permeabilized in 0.1% saponin, and processed for indirect immunofluorescence using the 12CA5 anti-HA antibody (*upper panels*), as described under "Materials and Methods." *C*, single-round kinetics of uptake and recycling of prebound biotinylated Tfn in tTA-HeLa cells uninduced (□) or induced to express either HA-Rab5(WT) (▲) or HA-Rab5(Q79L) (■) for 48 h as described under "Materials and Methods." Results are average ± S.D. of three independent experiments.

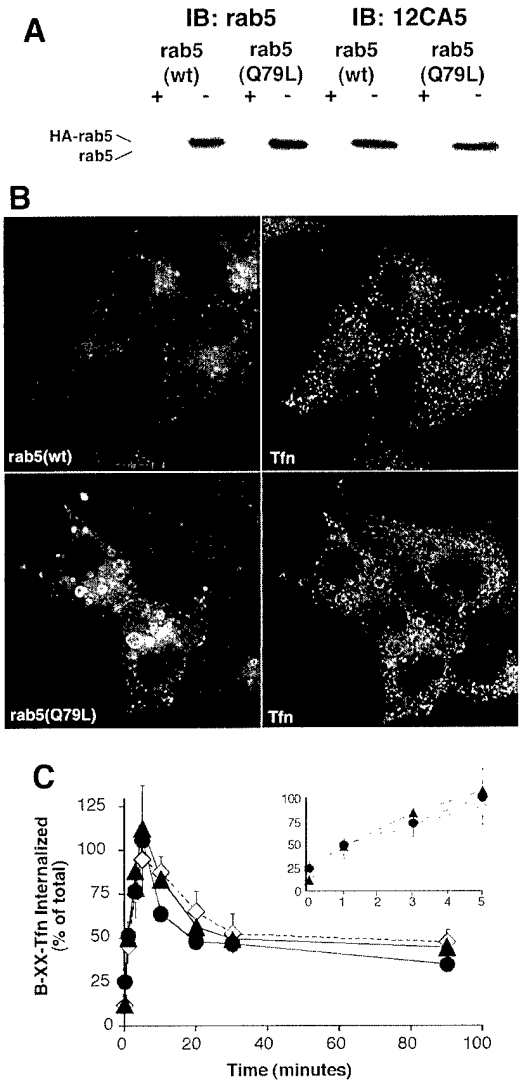

FIG. 2. **High levels of Rab5(Q79L) expression cause enlargement of endosomes without perturbing endocytic trafficking of transferrin.** tTA-HeLa cells infected with adenoviruses expressing either HA-Rab5(WT) or HA-Rab5(Q79L) under the control of a tetracycline-regulatable promoter were incubated for 18 h in the presence or absence of tetracycline as described under "Materials and Methods." *A*, immunoblots of cell lysates probed with either anti-Rab5 antibodies (*right panel*) or anti-HA antibodies (*left panel*). *B*, immunofluorescence images of adenovirally infected tTA-HeLa cells expressing either HA-Rab5(WT) or HA-Rab5(Q79L) incubated with Alexa-Tfn and subjected to indirect immunofluorescence with anti-HA monoclonal antibody as described under "Materials and Methods." *Left panels* show HA-Rab5 distribution visualized with a goat anti-mouse antibody conjugated to Texas Red; *right panels* show internalized Alexa-Tfn. *C*, single-round kinetics of uptake and recycling of prebound biotinylated Tfn in uninfected tTA-HeLa cells (◇) or in cells infected with either HA-Rab5(WT)-encoding adenoviruses (▲) or HA-Rab5(Q79L)-encoding adenoviruses (●) assayed 18 h after infection as described under "Materials and Methods." *Inset* shows expanded axis for early time points. Results are average ± S.E. of two independent experiments.

that recognizes the HA epitope (Fig. 2*B*). Despite the fact that transferrin receptors were trafficking through these dramatically enlarged endosomes when the kinetics of transferrin receptor trafficking was measured, there were no changes in the uptake of the transferrin receptor (Fig. 2*C*) compared with control uninfected cells or cells infected with HA-Rab5(WT). Like the stably transfected Rab5 tTA-HeLa cell lines, adenovirus expression of wild type and mutant Rab5 did not alter the steady state accumulation of Tfn within the cell.

Transferrin Endocytosis and Recycling Are Unaffected by HA-Rab5(Q79L) Overexpression—Our findings are inconsistent with previous results showing effects of both Rab5(WT) and Rab5(Q79L) overexpression on endocytosis and recycling of TfnR (5, 8). One trivial explanation for these differences is that previous studies were performed on adherent cells following internalization of ^{125}I-Tfn, whereas our assay follows B-XX-Tfn uptake in nonadherent cells. However, similar results were obtained when we assayed endocytosis and recycling of ^{125}I-Tfn in adherent adenovirally infected HeLa cells using the methodology of others (Refs. 5, 8, and data not shown). A second methodological difference was that previous studies employed a protocol that ensured a rapid bolus of Rab5 overexpression (5, 8). In contrast, the persistent overexpression of Rab5(Q79L) in our system may enable induction of a compensatory mechanism(s) that restores transferrin receptor trafficking to normal steady state rates. Therefore, we adapted our expression system for rapid induction of a bolus of protein expression. For these experiments, cells were infected with a 30-fold higher

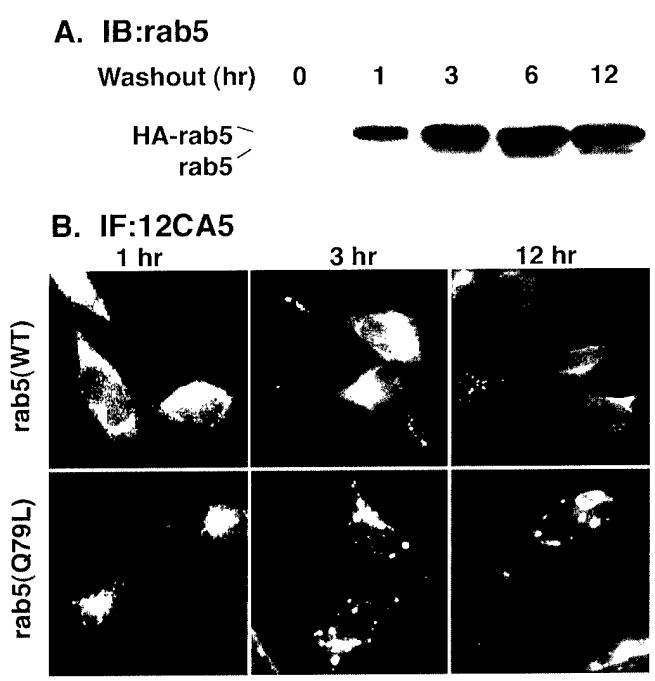

FIG. 3. **Bolus of Rab5 expression in adenovirally infected tTA-HeLa cells.** Rapid, high level expression of HA-Rab5(Q79L) in adenovirally infected tTA-HeLa cells was induced after release from a cycloheximide block as described under "Materials and Methods." *A*, immunoblot probed with an anti-Rab5 monoclonal antibody showing rapid induction of Rab5 expression detectable in 100 μg of whole cell lysates after cycloheximide wash out for the indicated times. *B*, adenovirally infected tTA-HeLa cells induced to express HA-Rab5(WT) or HA-Rab5(Q79L) by release from cycloheximide block for the indicated times were fixed and processed for indirect immunofluorescence (*IF*) using anti-HA antibody 12CA5. Results shown are representative of at least three independent experiments.

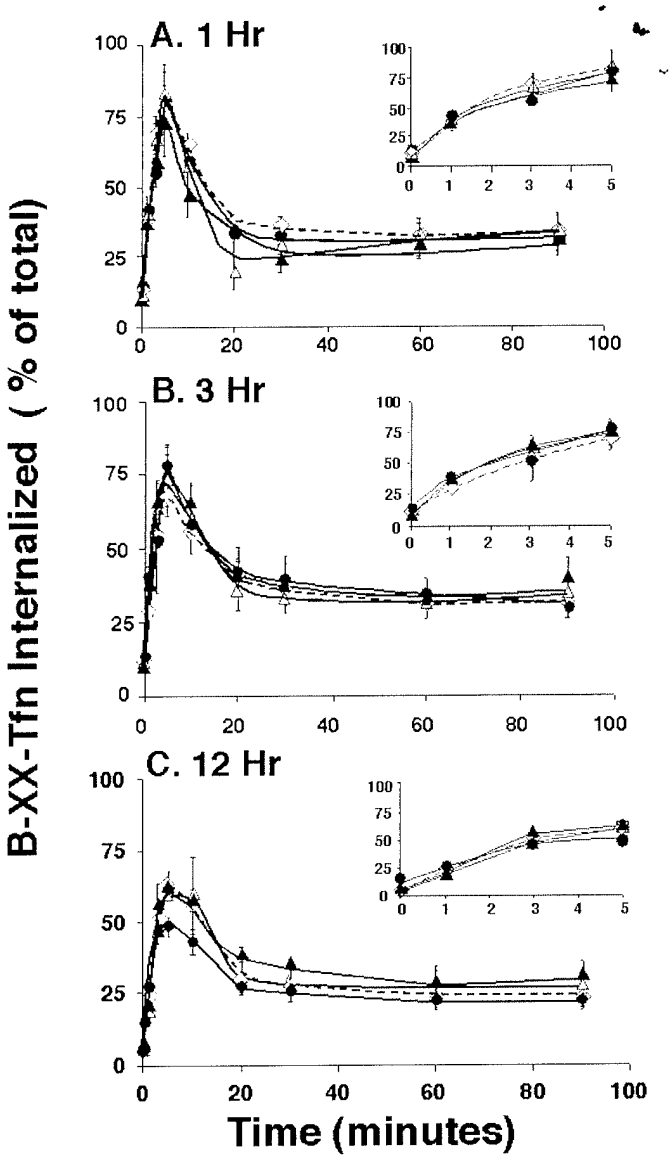

FIG. 4. **Endocytic trafficking of transferrin is unaffected even by rapid induction of high levels of Rab5(Q79L) expression.** Kinetics of uptake and recycling of prebound biotinylated Tfn were measured in uninfected tTA-HeLa cells (◇), tTA-HeLa cells infected with recombinant adenovirus that encode HA-Rab5(Q79L) but maintained in the presence of 1 μg/ml tetracycline (△), or tTA-HeLa cells infected with adenovirus in the presence of cycloheximide and induced to express either HA-Rab5(WT) (▲) or HA-Rab5(Q79L) (●) after release from cycloheximide for 1, 3, and 12 h as described under "Materials and Methods." Transferrin endocytosis and recycling were examined using an enzyme-linked immunosorbent assay-based assay, which monitors the total level of internalized, prebound, and biotinylated transferrin as described under "Materials and Methods." Data are plotted as a percentage of the total biotinylated transferrin initially bound to the cells. The same data are shown over a shorter time course (*inset*).

m.o.i. of adenovirus (see "Materials and Methods" for details), and Rab5 expression was controlled using cycloheximide. Briefly, tTA-HeLa cells were infected with adenovirus (m.o.i. of 300) for 2 h. Cells were then treated with 10 μg/ml cycloheximide for 3 h to accumulate mRNA in the absence of protein expression. The cycloheximide was washed from the cells, and protein was expressed for the indicated periods of time before experiments were performed. As shown in Fig. 3*A*, this protocol allows for tight control of protein synthesis while permitting a regulatable, high level of Rab5 expression. Within 1 h of HA-Rab5(Q79L) expression, exogenous HA-Rab5(Q79L) levels were estimated to be 50-fold over endogenous Rab5 (Fig. 3*A*). The level of HA-Rab5(Q79L) expression continued to increase with increased time of incubation in the absence of cycloheximide, plateauing at 6–12 h postinfection. A similar expression pattern was seen when cells were infected with HA-Rab5(WT) adenovirus (data not shown). After only an hour of protein synthesis, the enlarged endosomal phenotype could be detected (Fig. 3*B*). Increased duration of HA-Rab5(Q79L) synthesis caused a successive increase in the size of early endosomes, whereas increased HA-Rab5(WT) expression caused only minor increases in endosome size.

At each time point, we performed a kinetic analysis of transferrin uptake and recycling (Fig. 4). Consistent with our findings thus far, despite the dramatic changes in endosomal morphology seen at even the earliest time points of HA-Rab5(Q79L) expression (1 h), we were unable to detect changes in endocytosis or steady state accumulation of TfnR within the cell. Although endosome size continued to enlarge at 3 and 12 h of HA-Rab5(Q79L) expression, there was similarly no effect on the rates or efficiency of TfnR uptake and intracellular accumulation compared with uninfected cells or cells infected in the presence of 1 μg/ml tetracycline, which served as controls. In all cases, Tfn uptake was maximal at 5 min, and recycling occurred with a half-time of ~7–8 min, consistent with results of others (18–20).

Rates of Bulk Phase Endocytosis and Lipid Recycling Are Unaffected by Trafficking through Enlarged Endosomes—It has been proposed (1, 2) that sorting in the early endosome occurs, at least in part, by a default mechanism based on the geometry of the tubulovesicular early endosome. In this model, the high surface area of the tubular portions of the endosome facilitates recycling of membrane-associated components, perhaps through an iterative process (19, 21). Others have argued

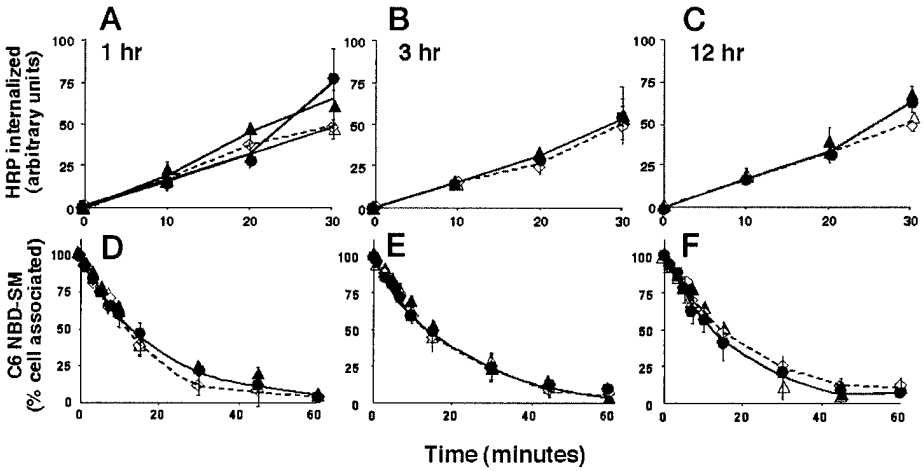

FIG. 5. **Overexpression of Rab5(WT) or Rab5(Q79L) for short or longer periods of time does not effect fluid phase uptake or lipid recycling.** Bolus expression of HA-Rab5(WT) (▲) or HA-Rab5(Q79L) (●) was induced for the indicated times in tTA-HeLa cells as in Fig. 3. As controls, cells were uninfected (◇) or infected with HA-Rab5(Q79L) adenovirus and cultured in the presence of tetracycline (△). *A–C*, the kinetics of fluid phase uptake of HRP at 37 °C measured as described under "Materials and Methods" and expressed in arbitrary units normalized to cellular protein and relative to control uninfected cells (n = 3, average ± S.E.). *D–F*, C_6-NBD-SM was internalized for 10 min and surface-associated lipid was removed. Shown are the kinetics of recycling of internalized lipid during subsequent incubation at 37 °C.

that more directed sorting mechanisms are required for the highly efficient endocytic trafficking of recycling receptors such as the TfnR (22). The appearance of coated buds containing TfnR on early endosomes (22) and the sorting motif-dependent inhibition of TfnR recycling by bafilomycin (23) support this latter hypothesis. Thus, our inability to detect an effect on the kinetics and efficiency of TfnR uptake and recycling in cells despite dramatic alterations in early endosome size and geometry may reflect the involvement of Rab5-independent, directed sorting events. Consistent with this possibility, one notable difference in our experiments compared with others is that in previous studies human TfnR vectors were introduced in parallel with the Rab5(WT) and Rab5(Q79L) constructs (8), whereas we are studying transport kinetics of endogenous receptors. Thus, it is possible that at higher levels of expression TfnR endocytic trafficking becomes more sensitive to alterations in endosomal morphology and/or Rab5 function than that of endogenous Tfn receptors. Importantly, we obtained similar results when examining endogenous TfnR endocytosis and recycling kinetics using adenovirally infected HepG2 cells expressing Rab5(Q79L) (data not shown).

To determine whether the dramatic changes in early endosome morphology affect the bulk sorting properties of early endosomes as predicted by current models, we examined the kinetics of endocytosis of a bulk fluid phase marker and the kinetics of recycling of a bulk membrane lipid marker. To focus on the rates of volume endocytosis rather than the extent of volume accumulation, we analyzed the initial rate of fluid phase HRP uptake. As can be seen (Fig. 5, *A–C*), we were unable to detect differences in the rate of HRP endocytosis at either 1, 3, or 12 h after bolus induction of expression of WT or mutant Rab5 compared with either uninduced or uninfected controls. Previous studies on HRP uptake in Rab5-expressing cells (7) focused on later time points of uptake when changes in endosomal volume will be reflected by increased accumulation of HRP at steady state. Our results suggest that GTP hydrolysis by Rab5 is not rate-limiting for bulk or receptor-mediated endocytosis in HeLa cells.

We next measured the rates of bulk membrane recycling in cells overexpressing Rab5(Q79L), expecting that membrane lipids would accumulate in the enlarged vacuolar portions of the early endosome slowing their recycling. For these experiments we used C_6-NBD-SM, a readily extractable, fluorescently labeled membrane lipid (17). Briefly, cells were labeled with C_6-NBD-SM for 10 min at 37 °C to allow the C_6-NBD-SM to traffic to the early endosomes. After extracting the plasma membrane C_6-NBD-SM through a series of backwashes in a fatty acid-free bovine serum albumin solution, dissociable C_6-NBD-SM was measured by the fluorescence in the medium. Unexpectedly, there was no appreciable difference in the rate or extent of C_6-NBD-SM recycling from the endosome to the plasma membrane at any time point after induction of Rab5 WT or mutant overexpression (Fig. 5, *D–F*). Thus, in these cells efficiency of recycling of either bulk membrane or TfnR was not affected by dramatic changes in endosome geometry.

Conclusions—We find that the early endosomal compartment significantly expands in cells overexpressing the constitutively active Rab5 mutant, Rab5(Q79L). This finding is consistent with previous work of others (8, 24) and with the model that Rab5 plays a critical role in early endosome biogenesis and morphogenesis by controlling the rate of endosome fusion events while in the Rab5/GTP-bound form (4, 5). Unexpectedly, and in contrast to previous reports (7, 8), in the presence of Rab5(Q79L) overexpression we observed no detectable acceleration in the kinetics of Tfn receptor or fluid phase uptake. Further, there were no changes in steady state, intracellular accumulation of the Tfn receptor or lipid recycling despite the appearance of these morphologically altered early endosomes. Our results were obtained at a variety of levels of Rab5(Q79L) overexpression, which caused varying degrees of change in endosome morphology and after even brief exposure to mutant Rab5 provided little opportunity for the induction of compensatory mechanisms.

Overexpression of dominant-negative Rab5 mutants (*e.g.* Rab5(N133I) or Rab5(S34N)) has been shown by several groups to inhibit TfnR and fluid phase endocytosis and endosome fusion (5, 8). These mutations block Rab5 function and can exert their inhibitory effects independently of downstream effectors. By contrast, the activating mutant studied here, Rab5(Q79L), will require interaction with downstream effectors to manifest its effects. Cell type and other variables may determine whether specific downstream effectors of Rab5 are limiting and therefore whether overexpression of Rab5(Q79L) will alter the kinetics of membrane trafficking along the endocytic recycling pathway. Thus, our results do not rule out a function for Rab5 in controlling membrane trafficking through the early/sorting endosome.

Regardless, the important findings from these studies are

2-fold. First, our results clearly establish that Rab5-induced changes in early endosomal morphology are not predictive of defects in endocytic membrane trafficking. Second, our results argue that geometric considerations may contribute to a lesser extent than previously assumed in determining the bulk sorting and recycling efficiencies of the early endosomal compartment. Morphometric measurements made of the tubular and vesicular portions of early endosomes in baby hamster kidney cells (25) show that 50–70% of total endosomal volume and 55–90% of total surface area are associated with the tubular portions of the endosome. Although there is considerable inherent error in these measurements (25), they also suggest that endosomal geometry would not be sufficient to account for the observed efficiency of sorting and recycling.

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Appendix C

Abstract from the 2001 American Association of Cancer Researchers Meeting

Expression of Rab(Q79L)-Induced Enlarged Endosomes Does Not Alter Epidermal Growth Factor Receptor Endocytic Trafficking

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As part of its molecular physiology, the EGFR enters the endocytic pathway upon ligand binding and subsequent activation. It has been demonstrated by numerous investigators that EGFR entry into the endocytic pathway is required for activation of some, but not all, EGFR mediated signal transduction. In an effort to further explore the role of the endocytic pathway in EGFR signal transduction, we examined EGFR trafficking in to and out of the early endosome with the goal of being able to explore EGFR signal transduction from that intracellular compartment. Rab5 is a small molcular weight guanine nucleotide binding protein involved in the formation of the early endosome. Expression of a constituitively active form of rab5 (glutamine to alanine mutation at amino acid residue 79 – denoted rab5(Q79L)) has been reported to induce the formation enlarged endosomes and result in the net accumulation of the transferrin receptor in the early endosome. Since this phenotype mirrored our desired results with the EGFR, we engineered a tetracycline-regulatable adenoviral expression system to introduce both wild type and constituitively active mutant rab5 into HepG2 cells. Overexpression of rab5(Q79L) produced the characteristic enlarged endosomal morphology. These endosomes could effectively traffic EGFR as evidenced by indirect immunofluorescent staining of the EGFR in the enlarged endosome after EGF treatment. However, there was no net accumulation of the EGFR in the endosomes as indicated by the unaltered rates of 125I-EGF endocytosis and recycling. Thus, rab5 is plays a role in the trafficking of EGFRs, but does not exert the same regulatory component in endocytosis that has been demonstrated with the transferrin receptor.

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Appendix D

List of Personnel receiving funding from DAMD17-99-1-9367

1. Brian P. Ceresa, Ph.D. – Principle Investigator
2. Jennifer Dinneen – Research Technician
3. Michael D. Franklin – Laboratory Technician
4. Jason Farris – Research Technician